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Comparative analysis of proteinase activities of *Bacillus thuringiensis*-resistant and -susceptible *Ostrinia nubilalis* (Lepidoptera: Crambidae)

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Abstract

Proteinase activities were compared in soluble and membrane fractions of guts obtained from larvae of *Bacillus thuringiensis*-resistant and -susceptible *Ostrinia nubilalis*. Overall, serine proteinases from soluble fractions of the susceptible strain were more active than those of the resistant strain. The soluble trypsin-like proteinase activity of the resistant strain was approximately half that of the susceptible strain. The number and relative molecular masses of soluble and membrane serine proteinases were different. However, there were no significant differences in the activities of serine proteinases and aminopeptidases extracted from midgut membranes of the two strains. Cry1Ab protoxin hydrolysis by soluble proteinase extracts of the resistant strain was reduced approximately 20–30% relative to that of the susceptible strain. Reduced protoxin processing due to decreased activities of Bt protoxin activation proteinases may be associated with resistance to Bt toxin in this resistant strain of *O. nubilalis*.

Keywords: *Bacillus thuringiensis*; Cry proteins; European corn borer; *Ostrinia nubilalis*; Trypsin; Chymotrypsin; Elastase; Resistance mechanisms

1. Introduction

The insecticidal crystal proteins (Cry proteins) produced by *Bacillus thuringiensis* (Bt) are commonly used as environmentally-safe alternatives to synthetic pesticides. Genes encoding Cry proteins have been genetically engineered into several crops to increase host plant resistance to pests. Transgenic Bt crops expressing Cry proteins have been commercially grown in many countries and are highly effective against target pests (Shelton et al., 2002). However, the consistent high-level expression of Bt toxins in transgenic crops can promote the development of Bt resistance by pests and jeopardize their long-term success.

The mechanisms underlying Bt resistance are found in the sequential steps in the proposed mode of action of Bt toxins (Höfte and Whiteley, 1989; Knowles and Dow, 1993; Gill et al., 1992; Schnepf et al., 1998). Following the ingestion of Bt crystals by susceptible insects, protoxins are solubilized and hydrolyzed by gut proteinases to an active toxin form. Activated Cry proteins pass through the peritrophic membrane and bind to proteins in the brush border membrane of midgut epithelial cells (Ferré and Van Rie, 2002). Toxin binding is followed by events that lead to cell lysis and disintegration of the brush border membrane, and

Abbreviations: BAρNA, *N*α-benzoyl-L-arginine ρ-nitroanilide; BBMV, brush border membrane vesicle; BSA, bovine serum albumin; Bt, *Bacillus thuringiensis*; DTT, dithiothreitol; DMF, *N*, *N*-dimethylformamide; DMSO, dimethylsulfoxide; LρNA, L-leucine ρ-nitroanilide; SAAPFρNA, *N*-succinyl-ala-ala-pro-phenylalanine ρ-nitroanilide; SAAPLρNA, *N*-succinyl-ala-ala-pro-leucine ρ-nitroanilide; SDS PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis.

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eventually insect death. Any change in insect gut physiology that affects one or more steps in this process could prevent toxicity and lead to the development of resistant pest populations.

Most studies have focused on two steps in the mode of action: proteolytic activation of protoxin and binding of active toxin to receptors. Reduced binding of Cry proteins to midgut receptors has been associated with resistance in several strains of *Plodia interpunctella* (Van Rie et al., 1990; Herrero et al., 2001), *Plutella xylostella* (Ferré et al., 1991; Bravo et al., 1992; Tabashnik, 1994; Tabashnik et al., 1994; Escriche et al., 1995; Tang et al., 1996), *Heliothis virescens* (MacIntosh et al., 1991; Lee et al., 1995; Gahan et al., 2001), *Spodoptera exigua* (Moar et al., 1995), and *Leptinotarsa decemlineata* (Loseva et al., 2002). However, a loss of toxin binding was not associated with resistance to Bt in several insect species (Gould et al., 1992; Luo et al., 1997; Herrero et al., 2001). Therefore, other mechanisms of resistance may be operating in these insects.

Serine proteinases, such as trypsin, chymotrypsin, and elastase, are important in both the solubilization and activation of Bt protoxins (Dai and Gill, 1993; Milne and Kaplan, 1993; Martínez-Ramírez and Real, 1996; Oppert, 1999). In some insects, changes in these proteinases have been associated with resistance to Bt toxin (Oppert et al., 1994, 1996, 1997; Forcada et al., 1996). Oppert et al. (1994, 1996) reported that a strain of *P. interpunctella* resistant to *Bt* subsp. *entomocidus* HD-198 processed Bt protoxin at a slower rate than the parental susceptible strain. This resistant strain lacked a major gut proteinase involved in activation of Bt protoxin (Oppert et al., 1997), and the proteinase mechanism was responsible for about 90% of the total resistance to Cry1Ab (Herrero et al., 2001). Enzymes from a strain of *H. virescens* resistant to *Bt* subsp. *kurstaki* HD-73 were reported to activate the protoxin more slowly and degrade the toxin faster than enzymes from a susceptible strain (Forcada et al., 1996, 1999). In *Spodoptera littoralis*, increases in the specific activity of gut proteinases were associated with the loss of sensitivity to Cry1C due to an increase in the degradation of active toxin (Keller et al., 1996).

Aminopeptidase is an exopeptidase and a marker for membrane proteins. It is localized to the brush border membrane of midgut epithelial cells and is involved in the digestion of peptides and amino acid transport (Terra and Ferreira, 1994). A membrane-bound glycosyl-phosphatidylinositol anchored aminopeptidase N has been reported to bind Cry1Ac toxins in several different insects (Sangadala et al., 1994; Gill et al., 1995; Knight et al., 1995).

Previously, a strain of Bt-resistant *Ostrinia nubilalis*, selected for resistance to *Bt* subsp. *kurstaki* (HD-1), was described with lower trypsin-like proteinase activity compared to the parental susceptible strain (Huang et al., 1999). In this study, we found that the reduced trypsin-like proteinase activity was in the soluble fraction of gut proteinases in the resistant strain. Soluble proteinases from the resistant strain incubated with Cry1Ab protoxin resulted in lower amounts of an active toxin fragment relative to incubations with proteinases from the susceptible strain. This reduction in toxin activation may account for lower susceptibility to Bt toxins by resistant *O. nubilalis* larvae.

2. Materials and methods

2.1. Insect strains

A susceptible strain of *O. nubilalis* was established from egg masses collected from cornfields near St. John, Kansas during 1995 and has been reared on artificial diet for over 40 generations (Reed et al., 1972; Huang et al., 1997). A Bt-resistant strain (KS-SC) has been selected from this susceptible strain for more than 40 generations by exposing neonates to a diet containing doses of *Bt* subsp. *kurstaki* HD-1 (Dipel®, Abbott Laboratories, Chicago, IL) that induced 80–95% mortality (Huang et al., 1997).

2.2. Bt toxin

Cry1Ab protoxin was obtained by the fermentation of recombinant *E. coli* ECE 54 (provided by *Bacillus* Genetic Stock Center, Department of Biochemistry, Ohio State University) and partially purified as previously described (Ge et al., 1990). Trypsin-activated Cry1Ab was purified with an Eco-Pac HQ anion column (Bio-Rad, Hercules, CA) as described by Hua et al. (2001).

2.3. Soluble proteinase extraction and pH measurements

Soluble proteinase extracts were prepared as previously described (Oppert et al., 1996) with slight modifications. Five fifth instars were weighed and chilled on ice. After the posterior and anterior ends of the larvae were removed, guts containing the food bolus were excised, immediately submerged in 50 μl of ice-cold distilled deionized water, and stored at $-80\ ^{\circ}C$ until use. Prior to assays, gut samples were thawed on ice, vortexed for 2 min, and spun at $15,000\times g$ for 5 min. Supernatants containing 1.2–1.8 mg of soluble protein were used in assays.

The pH of the anterior, middle, and posterior section of the midgut were made using a microelectrode (Microelectrodes, Inc., Bedford, NH) inserted directly into each part of the midgut. Hemolymph pH measurements were made with the same probe after collecting hemolymph from severed prolegs of the larvae.

2.4. Membrane proteinase extraction

Brush border membrane vesicle (BBMV) preparations were obtained from fifth instars as described by Wolfersberger et al. (1987). One to two milligrams of membrane proteins was obtained from midgut tissue of 100 fifth instars. Samples were separated into 50-μl aliquots, frozen in liquid nitrogen, and stored at −80 °C.

2.5. Protein assays

Protein concentrations in the samples were determined by the Bradford (1976) method using the Coomassie® Plus Protein Assay (Pierce, Rockford, IL), with BSA as a protein standard.

2.6. Proteinase activity assays

Total proteolytic activities of soluble and membrane serine proteinases were measured using fluorescently labeled casein (BODIPY TR-X, Molecular Probes, Eugene, OR) as a substrate in universal buffers at nine pH values ranging from 4.25 to 11.64 (Frugoni, 1957). Five microliters of soluble or membrane extracts was added to individual microtiter plate wells containing 85 μl of universal buffer at each pH value. Enzyme solutions were incubated with 10 μl of fluorescently labeled casein (10 μg/ml) at 37 °C for 4 h, and the relative fluorescence values were measured at excitation/emission wavelengths of 584/620 nm with a fluorescent plate reader (Fluoroskan Ascent FL, Thermo LabSystems, Beverly, MA). Incubation of substrate alone was used to correct the readings. The rate of caseinolysis was expressed as the relative fluorescence per mg of protein.

Specific activities of soluble and membrane serine proteinases were assayed with synthetic substrates in 100-μl reaction mixtures containing universal buffer of pH 11.1. *N*α-benzoyl-L-arginine ρ-nitroanilide (BAρNA) was used to evaluate trypsin-like proteinases, *N*-succinyl-ala-ala-pro-phenylalanine ρ-nitroanilide (SAAPFρNA) for chymotrypsin-like proteinases, and *N*-succinyl-ala-ala-pro-leucine ρ-nitroanilide (SAAPLρNA) for elastase-like proteinases. Soluble or membrane extracts of the resistant and susceptible strains were diluted to 1.0 mg/ml with universal buffer. Ten microliters of enzyme was added to each microtiter plate well containing 40 μl of buffers for assays of trypsin- and chymotrypsin-like proteinases, whereas 50 μl of enzyme was used to assay elastase-like proteinases. Previously prepared stock substrates of BAρNA (100 mg/ml in DMSO), SAAPFρNA (100 mg/ml in DMF), and SAAPLρNA (100 mg/ml in DMF) were diluted to 1.0 mg/ml with universal buffer. Substrate solutions (50 μl) were added to individual wells containing enzyme, and the absorbance of the reaction product, nitroaniline, was measured at 405 nm in 15-sec intervals for 5 min (EL-340 plate reader and KC-3 software, Bio-Tek Instruments, Winooski, VT). Control wells containing substrate only were subtracted as background. Specific proteinase activities for the three substrates were calculated based on the molar extinction coefficient of 8700 M^{-1} cm^{-1} for nitroaniline, and expressed in μmol/min×mg protein.

Aminopeptidase activity in membrane fractions (aminopeptidase is an integral membrane protein) was assayed with 2 μl of BBMV preparations from resistant or susceptible strains added to individual wells containing 48 μl of buffer at each pH value (4.25–11.64). Fifty microliter of LρNA diluted in the appropriate pH buffers (1.0 mg/ml) was added to individual wells containing enzyme solutions. The absorbance was measured at 405 nm in 15-sec interval for 5 min.

All reaction readings were conducted in triplicate, and all assays were replicated three to four times. The proteinase activity data were statistically analyzed with the Student *t*-test to compare the differences between resistant and susceptible strains (SAS Institute, 1990).

2.7. Proteinase activity blot analyses

To examine the number and molecular mass of serine proteinases, activity blot analysis of soluble and membrane trypsin-, chymotrypsin-, and elastase-like proteinases was conducted according to the methods described by Oppert and Kramer (1998). Briefly, enzyme samples (35 μg/lane) were separated using 10–20% Tricine gradient gels (Invitrogen, San Diego, CA) with Tricine sample buffers at 4 °C. Separated proteins were transferred to nitrocellulose membranes in Tris–glycine transfer buffer. The membrane was incubated in 5 ml of buffer A (200 mM Tris, pH 8.0, 20 mM $CaCl_2$) containing 2.5 mg of BAρNA for trypsin-like proteinases, SAAPFρNA for chymotrypsin-like proteinases, and SAAPLρNA for elastase-like proteinases. Incubations were at 37 °C for 30–60 min with slow agitation. Liberated nitroanilide was diazotized by subsequent incubations of 5 min each in 0.1% $NaNO_3$ in 1.0 M HCl, 0.5% ammonium sulfamate in 1.0 M HCl, and 0.05% *n*-(1-naphthyl)-ethylenediamine in 47.5% ethanol. Blots were immediately scanned, placed in heat-sealed plastic bags, and stored at −20 °C.

2.8. Zymogram analysis

The number and molecular mass of digestive proteinases associated with the hydrolysis of Bt protoxin were examined using zymogram analysis with Cry1Ab protoxin and casein as substrates. Soluble proteins (18 μg/lane) were separated by SDS-PAGE (Laemmli, 1970) in duplicate 10–20% Tris–glycine gels and processed for zymogram analysis as described by

García-Carreño et al. (1993). After electrophoresis, gels were equilibrated at room temperature for 15 min in either zymogram developing buffer (Invitrogen, San Diego, CA) or 50 mM sodium carbonate (pH 9.6) containing 10 mM DTT. One gel was incubated with 10 ml of 2% casein in zymogram developing buffer, and the other with 10 ml of 3.8 μM Cry1Ab protoxin in sodium carbonate buffer for 3 h at 37 °C. After washing with water, the gel strips were stained with Coomassie brilliant blue and destained for visualization of clear zones corresponding to active enzymes. The band patterns, intensities, and areas of each band were compared (Odyssey Infrared Imaging System, LI-COR).

2.9. Cry1Ab proteolysis in soluble proteinases

To compare the solubilization and activation of Bt protoxin by digestive proteinases from resistant and susceptible *O. nubilalis* larvae, Cry1Ab protoxin processing was quantitatively analyzed. Cry1Ab protoxin was incubated with soluble proteinase extracts from resistant and susceptible *O. nubilalis* larvae at 1:25 (w/w) in buffer A with a total volume of 40 μl at room temperature (~24 °C) with for varying times. Processing was stopped by heating the samples at 95 °C for 10 min. Processed products were separated by 4–12% SDS-PAGE and blotted on nitrocellulose membranes. Western blot analysis was performed using rat polyclonal antiserum against Cry1Ac (1:5000), demonstrated to be cross reactive to Cry1Ab, and goat anti-rat IRDye 800 (LI-COR, Lincoln, NE) (1:5000). Cry1Ab toxin was quantitated using 1.56–50.0 ng of purified trypsin-activated Cry1Ab as standard (Odyssey Infrared Imaging System, LI-COR) (see Fig. 6A). The level of protoxin processing was estimated by multiplying the intensity and area of Cry1Ab bands. For comparison of processing rates, the amount of Cry1Ab at different activation levels was adjusted by multiplying a coefficient (1 for ~74-, 1.5 for ~64- and 2.0 for ~58-kDa bands, respectively, see Fig. 6B). The adjusted amounts of Cry1Ab were summed, and the sums were compared in extracts of resistant and susceptible insects at the same incubation times. The protoxin proteolysis experiment was repeated three times. To further compare the processing of activated toxin, previously purified trypsin-activated Cry1Ab was assayed using the same methods as described above.

3. Results

To provide background information on the gut physiology of *O. nubilalis* larvae, measurements of gut pH and total protein were obtained. The pH values of the midgut and hemolymph were measured. The anterior midgut had a pH of 9.8 ± 0.05, the middle midgut pH was 10.0 ± 0.07, and the posterior midgut pH was 9.8 ± 0.14 (mean ± S.D., $n = 4$). The pH of hemolymph was 6.8 ± 0.14. Each gut of a fifth instar *O. nubilalis* larva had ~300 μg of soluble protein, and ~15 μg of membrane protein was extracted from BBMVs.

3.1. Proteinase activity

There were no significant differences ($P > 0.05$) in the specific activities at each pH value of either soluble or membrane proteinases from Bt-resistant or -susceptible *O. nubilalis* larvae (Fig. 1). In acidic buffers, activities of soluble or membrane proteinases were low. As the pH increased, proteolytic activities increased rapidly. The maximum hydrolysis of casein by proteinases in either soluble or membrane fractions occurred at pH 11.1.

The specific activity of trypsin-like proteinases in the soluble fraction of Bt-resistant *O. nubilalis* larvae was significantly lower than that of the susceptible larvae ($P = 0.0135$) (Fig. 2A). There were no significant differences in the activities of soluble chymotrypsin- ($P = 0.3543$) or elastase-like proteinases ($P = 0.0516$) in resistant and susceptible larvae. There were also no significant differences in any of the serine proteinase activities from membrane fractions of Bt-resistant and susceptible larvae (Fig. 2B).

When LρNA was used as a substrate, the optimal pH for leucine aminopeptidase activity of BBMV preparations from resistant and susceptible *O. nubilalis* larvae was 8.5 (Fig. 3). At each pH, there were no significant differences in leucine aminopeptidase activities between Bt-resistant and -susceptible larvae ($P > 0.05$).

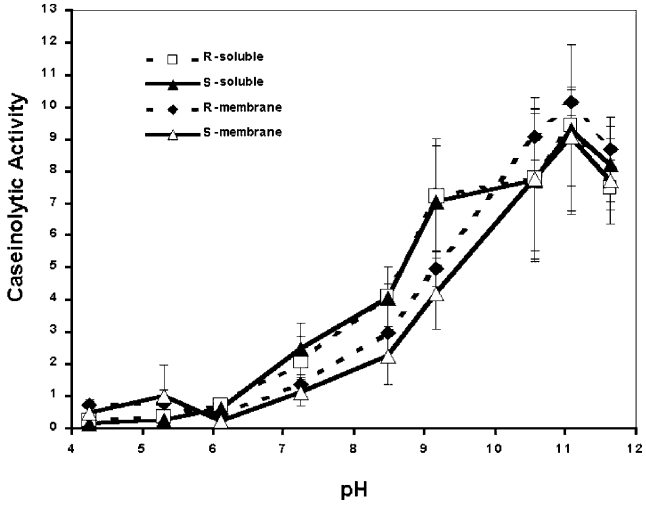

Fig. 1. Specific activity (hydrolysis of casein as measured by the relative fluorescence per mg protein) of digestive proteinases in soluble and membrane fractions from larvae of Bt-resistant (R) and -susceptible (S) *Ostrinia nubilalis*. Data are the mean ± S.D.

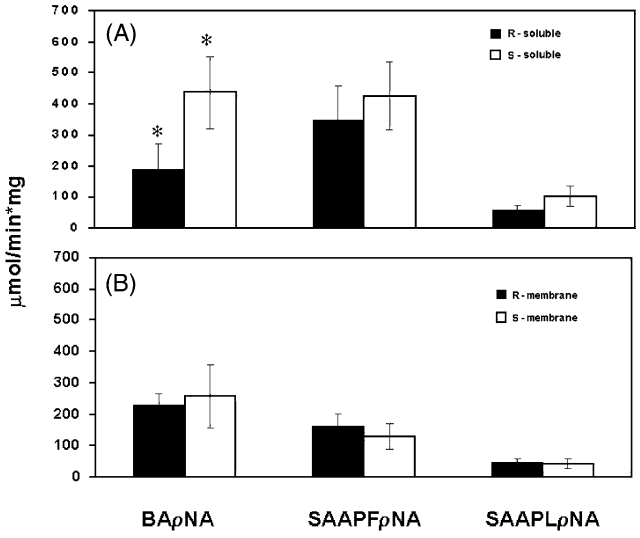

Fig. 2. Specific activities (μmol/min×mg) of trypsin- (using the substrate BAρNA), chymotrypsin- (SAAPFρNA), and elastase-like (SAAPLρNA) proteinases in soluble (A) and membrane (B) fractions from larvae of Bt-resistant (R) and -susceptible (S) *O. nubilalis*. Data are the mean ± S.D. Means with an asterisk are significantly different at the 5% level, as compared within each substrate.

3.2. Activity blots

The proteinase patterns of soluble trypsin-, chymotrypsin-, and elastase-like proteinases in the resistant and susceptible *O. nubilalis* larvae varied in intensity (Fig. 4). When BAρNA was used as a substrate, there were three bands with molecular masses of ~240 (A2), 85 (A3), and 34 (A4) kDa in soluble fractions of each strain. The intensities of A2, A3, and A4 bands were weaker in the resistant larvae than in susceptible larvae. In the membrane fractions, an additional band of tryptic activity was observed (Am1). The molecular mass of Am1 could not be estimated because the protein did not enter the resolving gel. When SAAPFρNA was used as a substrate, there were two bands with molecular masses of ~53 (F1) and 29 (F2) kDa in the soluble fractions of resistant and susceptible strains. The intensities of F1 and F2 were weaker in the resistant larvae than in susceptible larvae. A single SAAPFρNA hydrolysis activity (Fm2) with a molecular mass of ~34 kDa was observed in membrane fractions. No difference was observed in the intensity of Fm2 from the susceptible and resistant strains. When SAAPLρNA was used as a substrate, only one activity in soluble (L1, ~29 kDa) and membrane (Lm1, ~34 kDa) fractions from susceptible larvae was detected. In the soluble fraction from resistant larvae, L1 was resolved into two weaker activity bands (L_1 and L_2). The intensities of L_1, L_2, and Lm1 were weaker in the resistant larvae than in susceptible larvae.

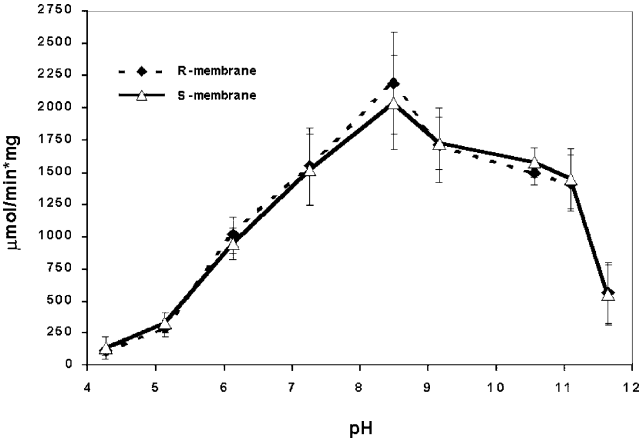

Fig. 3. Specific activities (μmol/min×mg) of aminopeptidases in membrane fractions from larvae of Bt-resistant (R) and -susceptible (S) *O. nubilalis*. Data are the mean ± S.D.

3.3. Zymogram

Using casein as a substrate, zymogram analysis indicated six proteinase activities (C1–C6) in soluble extracts of susceptible larvae (Fig. 5A). Activities C5 and C6 were the most prominent proteinases in susceptible larvae. However, C6 was not detected in soluble extracts from resistant larvae. When Cry1Ab protoxin was used as a substrate, four activities, P1–P4, with molecular masses of ~55, 38, 28, and 23 kDa, respectively, were detected in both *O. nubilalis* strains (Fig. 5B). The relative intensities of all proteinases in the resistant strain were weaker than the corresponding activities in the susceptible strain, despite similar amounts of protein in each lane (Fig. 5C). The most dramatic reduction in protoxin hydrolysis by the proteinases from the resistant larvae was in P4, approximately 68% lower than that from the susceptible larvae. Overall, the level of protoxin hydrolysis in the resistant strain was approximately 21% lower than in the susceptible strain.

3.4. Cry1Ab proteolysis

When soluble proteinase extracts of *O. nubilalis* larvae were incubated with Cry1Ab protoxin, four proteolytic products were observed, corresponding to ~124-, 74-, 64-, and 58-kDa proteins (Fig. 6B). The 58-kDa protein was similar to trypsin-activated Cry1Ab. The proteolysis of Cry1Ab protoxin by digestive proteinases of both strains was rapid. After 15 sec of incubation at room temperature, the protoxin was partially hydrolyzed in both samples, resulting in 74- and 64-kDa proteins. A difference in the processing rate was observed between susceptible and

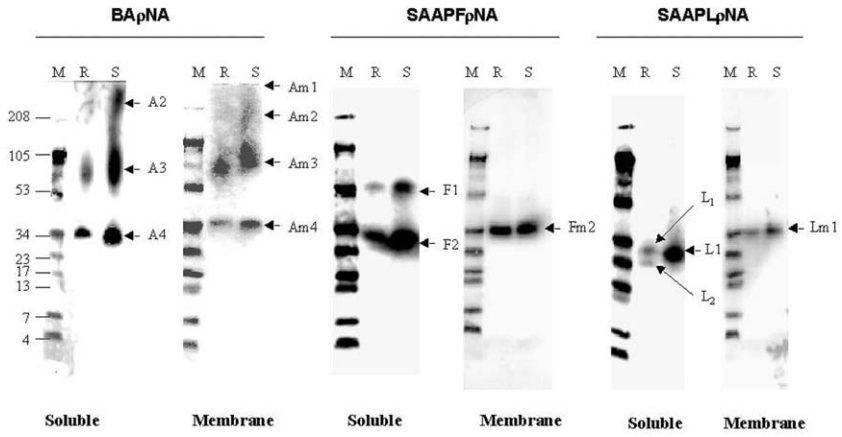

Fig. 4. Activity blots of trypsin- (BAρNA), chymotrypsin- (SAAPFρNA), and elastase-like (SAAPLρNA) proteinases in soluble and membrane-bound fractions from larvae of Bt-resistant (R) and -susceptible (S) *O. nubilalis*. M, molecular mass markers in kDa.

resistant larvae, based on the appearance of smaller proteins. Extracts from the susceptible strain produced a 58-kDa protein after a 15-sec incubation. There was no detectable protoxin after 360 min, and the toxin was hydrolyzed to the protease-resistant core, as demonstrated by a heavy band with a 58-kDa molecular mass. With extracts from the resistant strain, fully activated Cry1Ab (58-kDa band) was produced only after 30 min of incubation. Multiple intermediates were present even after 360 min incubation. Overall, Cry1Ab hydrolysis by proteinases of the resistant larvae was usually less than that of proteinases from the susceptible larvae (Fig. 6C). There was an average of 32% less activated Cry1Ab toxin in incubations with proteinases from the resistant larvae compared to those with susceptible larvae proteinases. There was no further proteolysis of trypsin-activated Cry1Ab protein with enzyme extracts from either strain in 180 min incubations (Fig. 6D).

4. Discussion

Soluble trypsin-like proteinase activity was significantly lower for Bt-resistant *O. nubilalis* larvae than for the susceptible larvae. This result agrees with previously reported data on trypsin-like proteinase activity using kinetic analyses (Huang et al., 1999). When casein was used as a substrate, there was no significant difference in the overall activity of the digestive proteinases for either the soluble or membrane fractions from larvae of resistant and susceptible *O. nubilalis*. In contrast, when Cry1Ab protoxin was used as a substrate, zymogram analysis indicated that ~20% less protoxin was hydrolyzed with soluble extracts from the resistant strain when compared with similar extracts from the susceptible strain. These results were corroborated by decreased temporal activation of Cry1Ab protoxin with soluble proteinases from the resistant strain when compared to that of the susceptible strain. One hypothesis is that reduced protoxin processing is

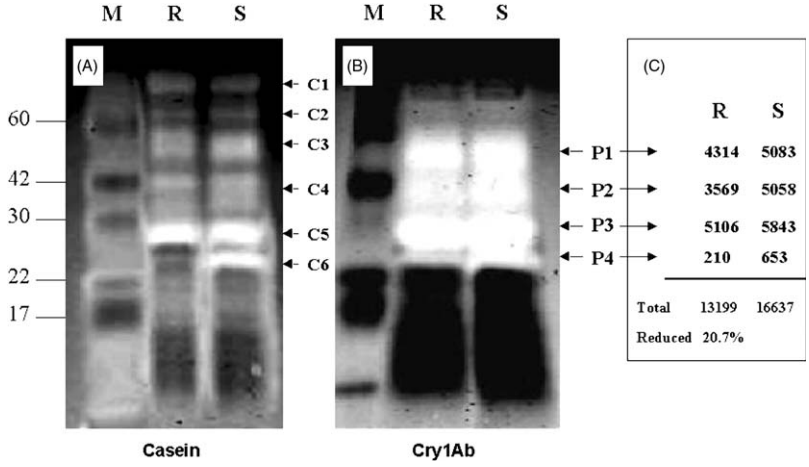

Fig. 5. Zymograms of soluble proteinases from larvae of Bt-resistant (R) and -susceptible (S) *O. nubilalis*. Substrates casein (A) and Cry1Ab protoxin (B) were used. Densitometric comparison of hydrolyzed-Cry1Ab bands of R and S strains is illustrated (C) (unit: pixel).

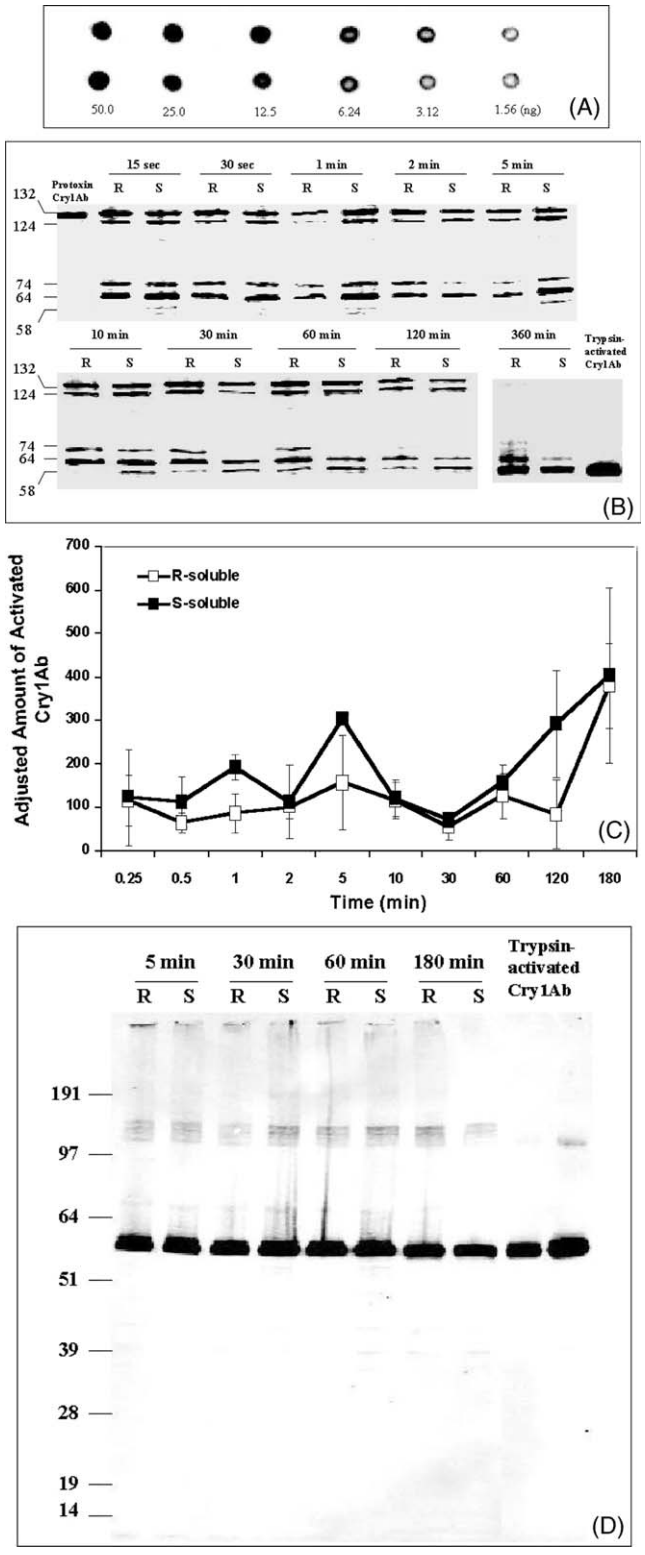

Fig. 6. Processing of Cry1Ab with soluble proteinase extracts from larvae of Bt-resistant (R) and -susceptible (S) *Ostrinia nubilalis*. (A) Purified trypsin-activated Cry1Ab was used as a quantification standard; (B) Processing of Cry1Ab protoxin was compared between R and S strains at varying times; (C) Amounts of Cry1Ab protoxin activated by gut extracts from R and S larvae were compared (mean ± S.D.). (D) Processing of purified trypsin-activated Cry1Ab was compared. Molecular masses in kDa are shown on the left.

important in enhancing the insects' capacity to withstand larger Bt protoxin doses and thus provide a survival advantage. Results observed in vitro may be different from in vivo, where the solubilization, activation, binding, and insertion events progress in a dynamic fashion. In the gut, the reduction in protoxin hydrolysis may have a greater effect on the insect response to Bt.

Research is needed to understand the molecular mechanisms of decreased proteinase activity in this resistant *O. nubilalis* strain. The resistant strain is ~70-fold resistant to Dipel (Huang et al., 1997), and ~200-fold resistant to Cry1Ab and ~500-fold resistance to Cry1Ac protoxins (unpublished data). Binding properties of Cry1Ab and Cry1Ac to BBMVs from the resistant and susceptible strains using ligand blot analysis, surface plasmon resonance assays, and radiolabeled toxin binding assays indicated that toxin binding affinity, binding site concentration, or overall binding capacity were similar in the resistant and susceptible strains (unpublished observations). Proteomic comparison of Bt-susceptible and -resistant strains of *Plodia interpunctella* provided evidence that proteinase-mediated resistance was associated with multiple protein alterations related to increased oxidative metabolism (Candas et al., 2003). Experiments to determine whether the reduction in protoxin hydrolysis in Bt-resistant *O. nubilalis* is responsible entirely for resistance to Bt are in progress.

In this study, we found that the pH of the midgut varied from 9.8 to 10, while the pH of the hemolymph was 6.8. This is similar to the pH values reported for other Lepidoptera (Terra et al., 1996; Dow, 1984). The pH for optimal proteinase activity in the gut varies significantly among different taxa of insects and among individual proteinases (Wolfson and Murdock, 1990). For lepidopteran insects, the typical pH for optimal protein proteolysis occurs at alkaline pH values ranging from 9.8 to 11.2. The optimum pH for proteinase activity of either soluble or membrane fractions from *O. nubilalis* larvae was 11.1 when casein was used as the substrate. Our results are consistent with previously published data regarding *O. nubilalis* and other lepidopteran insects (Houseman et al., 1989; Houseman and Chin, 1995; Bernardi et al., 1996; Huang et al., 1999). When LρNA was used as a substrate, the optimal pH for leucine aminopeptidase activity of membrane fractions of resistant and susceptible *O. nubilalis* larvae was 8.5, which is similar to the aminopeptidase activity of other lepidopteran insects, such as *Bombyx mori* (Parenti et al., 1997).

Increased aminopeptidase activity has been associated with Bt resistance in some insects (Zhu et al., 2000; Loseva et al., 2002). In a Bt-resistant strain of *P. interpunctella*, aminopeptidase-like mRNA expression levels were slightly increased (Zhu et al., 2000). Elevated

aminopeptidase activity was also reported in a strain of *L. decemlineata* resistant to Cry3Aa (Loseva et al., 2002). However, the aminopeptidase activities in neonates and third instar larvae of *Manduca sexta*, *Pieris brassicae*, *Mamestra brassicae*, and *Agrotis ipsilon* were inversely correlated with increased resistance during larval development (Gilliland et al., 2002). In this study, we did not find significant differences in the specific activities of aminopeptidases in resistant and susceptible *O. nubilalis*.

In BAρNA activity blot assays, an unresolved proteinase activity in samples from larvae of resistant or susceptible *O. nubilalis* was detected. This phenomenon is similar to the observation by Houseman and Chin (1995) where a high molecular weight aggregate with tryptic activity failed to migrate into the resolving portion of the electrophoretic gel.

Activated Cry1Ab was resistant to further proteolysis in vitro by proteinases from either resistant or susceptible larvae. However, four groups of active proteinases were observed in the Cry1Ab–zymogram analysis, suggesting that the protoxin was completely hydrolyzed in these areas. Although, the toxin core is resistant to proteolysis, this resistance can be overcome by an excessive amount of proteinases under favorable reaction conditions, such as the case of localized areas of proteinases in an SDS-PAGE incubated in an optimal pH buffer under elevated temperature. Typically, insect gut proteinases cleave about 500 amino acids from the C-terminus of Cry1 protoxins and 28 amino acids from the N-terminus (Chestukhina et al., 1982; Choma et al., 1990). The cleavage sites of trypsin activity are at the C-terminus of the amino acid arginine and lysine. The final Cry1Ab protoxin hydrolysate product, which is the proteinase-resistant core protein, was ~58 kDa, produced by either soluble proteinase extracts or mammalian trypsin. Whether their N-terminal and/or C-terminal sequences are also the same is unknown. However, neither susceptible nor resistant proteinases were able to degrade a trypsin-hydrolytic core toxin, suggesting that removal of the activated form of the toxin from the gut was not a resistance mechanism in the Bt-resistant *O. nubilalis* larvae.

In summary, the soluble trypsin-like proteinase activity and Bt protoxin processing of a Bt-resistant *O. nubilalis* strain was reduced in comparison to that of a Bt-susceptible strain. Therefore, proteinase-mediated resistance to Bt protoxins is a possible resistance mechanism in this resistant *O. nubilalis* strain. Compared to receptor-mediated Bt resistance, proteinase-mediated Bt resistance may be more difficult to detect and more persistent in the environment, because it may be associated with lower levels of resistance, and multiple proteinase genes may be involved. If the proteinase activation of Bt protoxin is a contributing factor to Bt resistance development, transgenic plants producing full-length protoxin or even semi-truncated toxin at low to moderate levels may increase the possibility of resistance development.

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